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# An in vitro assay approach to investigate the potential impact of different doping agents on the steroid profile

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## Abstract

The steroid profile, that is, the urinary concentrations and concentration ratios of selected steroids, is used in sports drug testing to detect the misuse of endogenous steroids such as testosterone. Since several years, not only population-based thresholds are applied but also the steroid profile is monitored via the Athlete Biological Passport whereby the individual reference ranges derived from multiple test results of the same athlete are compared to population-based thresholds. In order to maintain a high probative force of the passport, samples collected or analyzed under suboptimal conditions should not be included in the longitudinal review. This applies to biologically affected or degraded samples and to samples excluded owing to the presence of other substances potentially (or evidently) altering the steroid profile. Nineteen different doping agents comprising anabolic steroids, selective androgen receptor modulators, selective estrogen receptor modulators, ibutamoren, and tibolone were investigated for their effect on the steroid profile using an androgen receptor activation test, an androgen receptor binding assay, an aromatase assay, and a steroidogenesis assay. The in vitro tests were coupled with well-established liquid chromatography/mass spectrometry-based analytical approaches and for a subset of steroidal analytes by gas chromatography/mass spectrometry. The variety of tests employed should produce a comprehensive data set to better understand how a compound under investigation may impact the steroid profile. Although our data set may allow an estimate of whether or not a substance will have an impact on the overall steroid metabolism, predicting which parameter in particular may be influenced remains difficult.

**KEYWORDS**
doping agents, GC/MS, H295R, LC/MS, steroidogenesis

## 1 | INTRODUCTION

In order to detect the misuse of anabolic–androgenic steroids (AAS) such as testosterone (T), sports drug testing methods rely on the measurements of urinary concentrations and concentration ratios of T and its metabolites as well as metabolically independent steroids like epitestosterone (E).[1] Sophisticated methods are necessary to differentiate between the endogenous production of the hormone and

administration of its synthetic copy. The World Anti-Doping Agency (WADA) has implemented the steroidal module of the Athlete Biological Passport in order to monitor endogenous hormone levels, thereby enabling an individual longitudinal steroid profile for each athlete.[1] Doping control laboratories worldwide contribute to each individual Steroid Passport (SP) over a prolonged time period; therefore, it is necessary to ensure the integrity of each data point in a steroid profile by enforcing strict regulations and harmonized analytical methods. Confounding factors (CFs) may have an impact on the SP and thus should be considered to avoid falsely classified samples.[2–4] For example, a prominent CF in sports drug testing is the ingestion of ethanol, which can lead to strongly elevated T/E ratios hours after consumption.[5,6]

In order to maintain the integrity of the SP, all samples showing the presence of any CFs should be excluded when justified. Whereas several CFs have already been identified, for instance, ethanol, selective estrogen receptor modulators (SERMs) or glucocorticoids,[5–9] for many doping agents, nutritional supplements, and nonapproved substances, their possible impact has not been investigated; and therefore, samples containing them are not currently excluded.

Owing to ethical and economic reasons, strategies other than in vivo studies of doping agents, nonapproved substances, and nutritional supplements are desirable. A cost-effective in vitro assay-based tool to evaluate the possible impact of any CFs on the SP would be particularly helpful, especially if a combination of complementary assays is considered. The aim of this research study was to investigate the probative force of a combination of different in vitro assays in order to evaluate the impact of a potential CF on the steroid profile.

The core part of the in vitro assay approach is represented by the steroidogenesis assay based on human adrenocortical carcinoma cells. Adrenal glands are the predominant site of steroid synthesis in the human body with site-specific production of glucocorticoids, mineralocorticoids, and precursors of active androgenic steroids. Therefore, a human adrenocortical carcinoma cell line (H295R) was utilized.[10–12] The potential of this cell line for steroid analysis was recognized by the Organisation for Economic Co-operation and Development (OECD) when testing potential endocrine disruptors.[13] The initial testing strategy and validation of the assay monitored the impact of chemical substances exclusively on the production of T and 17β-estradiol (E2).[13,14] Quantification of target analytes was commonly accomplished by means of immunological test methods due to the low concentration of both T and E2. Switching to liquid chromatography/mass spectrometry (LC/MS)-based methods allowed for broadening the scope of steroids under investigation.[15–18] Following the same intention, gas chromatography–mass spectrometry (GC–MS)-based methods have also been developed.[16,19,20] This current study also uses a full validated LC/MS-based method to measure T, E2, estrone (ES), progesterone (PROG), 17α-OH-progesterone (17OH-PROG), and cortisol (CORT). To test for a possibly increased sensitivity of the response of the H295R cell system to the potential CFs, a method based on GC–MS was developed to detect dehydroepiandrosterone (DHEA), T, androstenedione (ADION), pregnenolone (PREG), 17α-OH-PREG (17OH-PREG), 17OH-PROG, 11-deoxycortisol (11CORT), and CORT.

The in vitro assay approach also included screening for androgen receptor (AR) activation and aromatase inhibition. The CFs under investigation comprised AASs, selective AR modulators (SARMs), SERMs, a growth hormone secretagogue (GHS), and a selective tissue estrogenic activity regulator (STEAR) as listed in Table 1. Related chemical structures are depicted in Figure 1. The STEAR was of particular interest, as the impact of tibolone (TB) on the urinary steroid profile remains unclear in sports drug testing. As shown in Figure 1, TB is an orally administered synthetic steroid with a reported weak androgenic activity.[21,22] Clinically, its use for menopausal hormone therapy has been described. Since 2006, TB is included in the subgroup of "Other Anabolic Agents" on WADA's Prohibited List.[23,24] Although being explicitly forbidden in sports for over a decade with several adverse analytical findings being annually reported, little is known about the potential impact of this steroidal substance on the steroid profile as monitored in routine doping controls. Similarly, limited information is available for the majority of potential CFs investigated in this study.

## 2 | EXPERIMENTAL

### 2.1 | Reagents and chemicals

All reagents and chemicals were of analytical grade. Steroid reference materials PROG, ADION, and PREG were purchased from Serva (Heidelberg, Germany); and T, ES, 17OH-PROG, 17OH-PREG, CORT, 11CORT, and DHEA were from Sigma-Aldrich (Taufkirchen, Germany). Deuterated internal standards dehydroepiandrosterone-2,2,3,4,4,6-$d_6$ (DHEA-D6) and cortisol-9,11,12,12-$d_4$ (CORT-D4) were from Sigma-Aldrich; and testosterone-16,16,17-$d_3$ (T-D3) and methyltestosterone (MeT) were from LGC (Wesel, Germany). All chemical substances investigated for CF potential in the steroid profile samples are listed in Table 1.

### 2.2 | Compound preparation

Compounds were obtained as dry samples and diluted in dimethyl sulfoxide (DMSO) to yield stock solutions at 30 mM. The stock was serially diluted with DMSO in steps of three to yield concentrations from 30 mM to 2E−08 M. Compound solutions were added to the assays at an appropriate volume and dilution to yield concentrations ranging from 3E−5 to 2E−11 M and a DMSO concentration of 0.1%.

### 2.3 | AndroR transcriptional activation assay

Transcriptional activation mediated by the AR activation pathways was determined in PC3 cells stably transfected with a firefly luciferase gene under the control of the MMTV-LTR promoter.[25] Cells were seeded in 384-well plates (Greiner 781092, with lids) at 10,000 cells/well for a total volume of 50 μl/well; and 24 h later, test compounds diluted in media were added. After 21 h of further incubation, resazurin sodium

**TABLE 1** List of chemical substances under investigation as CFs in this study

| Name | Abbreviation | CAS number | Supplier | Drug class |
|---|---|---|---|---|
| Stanozolol | STAN | 10418-03-8 | Sigma-Aldrich[a] | AAS |
| Oral turinabol | DHCMT | 2446-23-3 | LGC[b] | AAS |
| Methylstenbolone | MSTEN | 6176-38-1 | LGC | AAS |
| Methandienone | MET | 72-63-9 | LGC | AAS |
| MK-677 | MK6 | 159752-10-0 | MedChemExpress[c] | GHS |
| RAD-140 | RAD | 1182367-47-0 | MedChemExpress[a] | SARM |
| Andarine | AND | 401900-40-1 | MedChemExpress[a] | SARM |
| MK-0773 | MK0 | 606101-58-0 | MedChemExpress[a] | SARM |
| LY2452473 | LY | 1029692-15-6 | MedChemExpress[a] | SARM |
| LGD-3303 | LGD3 | 917891-35-1 | MedChemExpress[a] | SARM |
| ACP-105 | ACP | 899821-23-9 | MedChemExpress[a] | SARM |
| GSK-2881078 | GSK | 1539314-06-1 | MedChemExpress[a] | SARM |
| LGD-2226 | LGD2 | 328947-93-9 | Sigma-Aldrich | SARM |
| PF-06260414 | PF | 1612755-71-1 | Sigma-Aldrich | SARM |
| TFM-4-AS-1 | TFM | 188589-61-9 | Sigma-Aldrich | SARM |
| LGD-4033 | LGD4 | 1165910-22-4 | Cayman Chemical[d] | SARM |
| Tibolone | TB | 5630-53-5 | LGC | STEAR |
| Clomifen (citrate) | CLOM | 50-41-9 | LGC | SERM |
| Tamoxifen (citrate) | TA | 54965-24-1 | LGC | SERM |

[a]Sigma-Aldrich (Taufkirchen, Germany).
[b]LGC (Wesel, Germany).
[c]MedChemExpress (Princeton, NJ, USA).
[d]Cayman Chemical (Ann Arbor, MI, USA).

salt (0.05 mg/ml final, Sigma R7017) was added to determine cell viability. Fluorescence intensity (excitation: 560 nm, emission 590 nm) was measured after 3 h of incubation. Cells were then lysed in luciferase substrate/lysis buffer (tricine pH 7.8 [40 mmol/L], $MgSO_4$*$7H_2O$ at 16 mmol/L, EDTA 0.2 mmol/L, DTT 15 mmol/L, coenzyme A free acid 0.25 mmol/L, luciferin 0.5 mmol/L, ATP 1 mmol/L, Tris pH 7.8 [50 mmol/L], $Na_2HPO_4$*$12H_2O$ 50 mmol/L, DTT 4 mmol/L, Triton X-100 6% [v/v], and glycerin 20% [v/v]), and luminescence was measured immediately afterwards. Dihydrotestosterone (5 nmol/L) and digitonin (0.3 mmol/L) were used as active control for AR activation and cell death, respectively, and DMSO (0.1% v/v final) as vehicle control.

## 2.4 | AndroR binding assay

AR binding was determined using a recombinant rat AndroR-ligand binding domain and a proprietary fluorescent androgen ligand (PolarScreen™ Androgen Receptor Competitor Assay Kit, Green, Thermo Fisher Scientific) following the manufacturer's instructions. The assay was performed in 384-well microplates (Greiner 781076) in a total volume of 20 µl/well. The reaction was started by combining premixed receptor and ligand with prediluted test compounds. Fluorescence polarization (excitation: 485 nm, emission 535 nm) was measured after 4 h of incubation. Dihydrotestosterone (10 µM) was used as active control and DMSO (0.1% v/v final) as vehicle control to define 100% and 0% displacement, respectively.

## 2.5 | Aromatase assay

Aromatase inhibition was determined using recombinant human aromatase (CYP19 + P450 Reductase Supersomes, Corning 456260, 7.5 nmol/L) with 7-methoxy-4-(trifluoromethyl)coumarine (Sigma T3165, 50 µmol/L) as substrate and glucose-6-phosphate, glucose-6-phosphate-dehydrogenase, and NADP+ as reducing-equivalent regenerating system in potassium phosphate buffer (50 mmol/L, pH 7.5 with $MgCl_2$ 0.4 mmol/L). The assay was performed in 384-well microplates (Greiner 781076) in a total volume of 25 µl/well. Fluorescence intensity (excitation: 409 nm, emission: 530 nm) was measured before the reaction to identify autofluorescent test compounds. The reaction was started by combining premixed cofactors with premixed enzyme, substrate, and test compounds. Fluorescence intensity was measured as above after 2 h of incubation. Letrozole (10 µM) was used as active control and DMSO (0.1% v/v final) as vehicle control to define 100% and 0% inhibition, respectively.

## 2.6 | Steroidogenesis assay

Test compound effects on steroidogenesis were determined in NCI-H295R cells following a modification of OECD TG No. 456 for high throughput.[13,18] Cells were seeded in 384-well plates (Greiner 781092, with lids) at 16,000 cells/well for a total volume of 50 µl/well in medium containing forskolin (10 µM). Test compounds

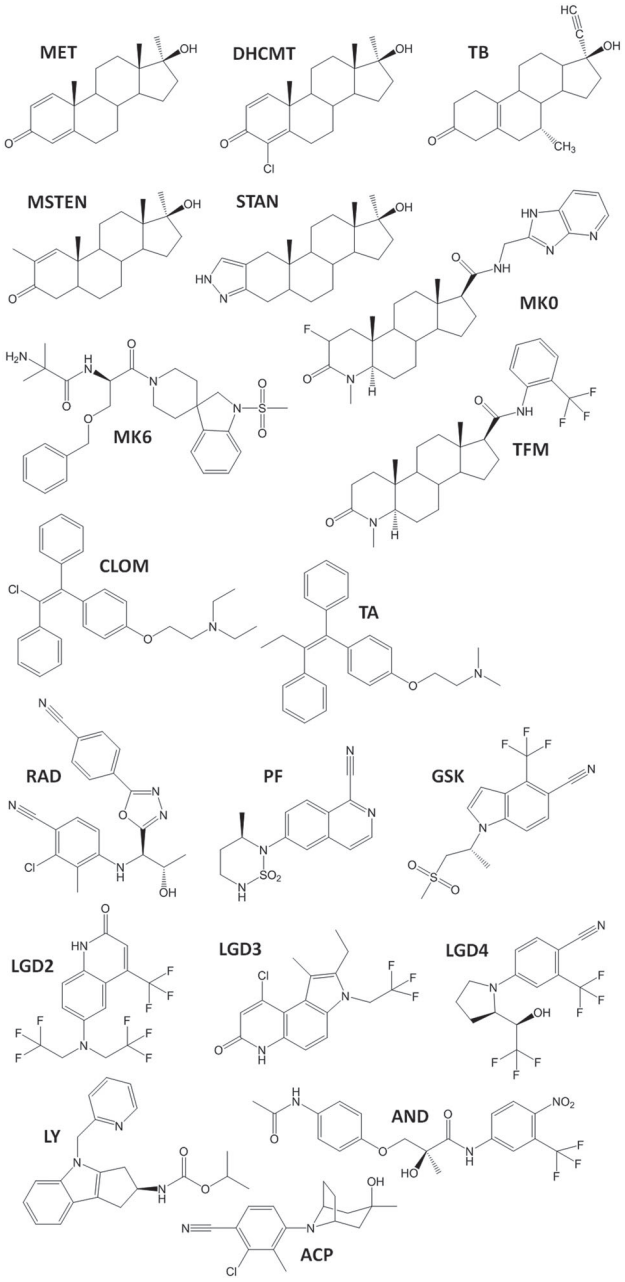


**FIGURE 1** Chemical structure of all compounds under investigation

diluted in media were added after 24 h, and following a further incubation step of 48 h, 40 µl of the supernatant was removed and precipitated with 160 µl of trichloroacetic acid and acetonitrile (4% w/v). Cell viability was determined with a proprietary nonlytic luciferase assay (Real Time Glo, Promega) according to the manufacturer's instructions. The previously removed supernatant was replaced with fresh medium containing the assay components. Luminescence was measured after a 4-h incubation period. Forskolin (10 µmol/L) and prochloraz (1 µmol/L) were used as active controls for activating and inhibitory effects on steroids, respectively, and DMSO (0.1% v/v final) as vehicle control. Digitonin (0.3 mmol/L) was used as active control for effects on cell viability. H295R maintenance culture was performed as specified in OECD TG No. 456.

## 2.7 | Liquid chromatography/mass spectrometry setup

The instrumentation used for all determinations was a Sciex 6500+ (Sciex, Darmstadt, Germany) coupled via an electrospray ionization (ESI) source to an Agilent 1290 II equipped with a Waters A HSST3 100 * 2.1 mm column. Analytes were separated by gradient elution in acetonitrile modified with 125 µl/L of formic acid beginning at 10% (v/v) and ending at 90% and high-purity water (Millipore) modified with 100 µl/L of formic acid beginning at 90% and ending at 10%. The column temperature was 60°C, the flow rate was 0.6 ml/min, and the total separation time was 3.6 min. The relevant steroids were quantified against their deuterated or $^{13}$C-labeled internal standards, which were added to each sample to reach a final concentration of 2 ng/ml and are listed in Table 2 together with the applied ion transitions.

## 2.8 | GC–MS setup

To simplify sample preparation, precipitation was carried out with pure cold ACN. After centrifugation, 120 µl of the supernatant was transferred into a new well plate and stored frozen at −18°C.

The ACN from each well was transferred into a conical test tube and evaporated to dryness after adding 10 µl of an internal standard mixture containing DHEA-D6, T-D3, and MeT at 1 ng/µl and CORT-D4 at 5 ng/µl. The dry residue was trimethylsilylated by adding 30 µl of a mixture containing MSTFA/$NH_4I$/ethanethiol 1,000:2:3 (v:w:v) and incubating for 30 min at 60°C in a heating block. After being cooled to room temperature, the residue of ca. 20 µl was transferred into auto-sampler vials with conical inserts and forwarded to GC/MS determinations.

The Agilent 7890 GC (Waldbronn, Germany) was equipped with an Agilent HP-Ultra 1 (17-m length, 0.2-mm inner diameter, 0.11-µm film thickness) and coupled to an Agilent 7000 triple quadrupole mass spectrometer. Injections were performed in pulsed splitless mode (40 psi for 0.5 min) with an injection volume of 4 µl at 300°C into an Agilent Inlet liner (Ultra Inert, splitless, single taper, equipped with glass wool). The temperature program started at 100°C and held for 2 min; then increased to 205°C at a ramp of 40°C/min, then to 280°C with 8°C/min, and finally with 40°C/min to 320°C; and held for 1 min before cooling. Carrier gas was helium (purity 4.6) at a constant pressure of 17.7 psi.

The mass spectrometer was operated in multiple reaction monitoring mode, and employed ion transitions are listed in Table 3. Nitrogen (purity 5.0) was used as collision gas at a flow of 1.5 ml/min, and the transfer line was set to 300°C. Data were collected and evaluated with Agilent Mass Hunter software B.07.

**TABLE 2** Summary of analytical parameters for target analytes measured by LC/MS

| Analyte | RT | Precursor ion | Product ion | Collision energy |
|---|---|---|---|---|
| CORT | 1.06 | 363 | 121 | 33 |
| CORT-D4 | 1.06 | 367 | 121 | 33 |
| E2 | 1.29 | 255 | 159 | 25 |
| E2-D5 | 1.28 | 260 | 161 | 25 |
| ES | 1.36 | 271 | 133 | 29 |
| ES-$^{13}$C3 | 1.36 | 274 | 136 | 29 |
| T | 1.35 | 289 | 97 | 27 |
| T-D3 | 1.34 | 292 | 97 | 38 |
| 17aPROG | 1.42 | 331 | 97 | 33 |
| 17aPROG-D8 | 1.42 | 339 | 100 | 33 |
| PROG | 1.62 | 315 | 97 | 29 |
| PROG-D9 | 1.62 | 324 | 100 | 33 |

*Note*: Listed are the retention times (RTs) and the applied precursor–product ion pairs (all measured at unit resolution).

### 2.9 | Software

Tibco Spotfire was used for generating graphs. Excel with the Excel-Fit add-in was used for conversion of raw data to percent inhibition or percent activation values and for calculating IC50 and AC50 values.

## 3 | RESULTS AND DISCUSSION

All potential CFs under investigation (Table 1) have been subjected to the different in vitro assays to evaluate their potential impact on the human steroid profile. Each in vitro assay will be presented individually to enhance the traceability of results. The results obtained for the potential CFs are summarized according to the different drug classes they belong to in anticipation of comparable results within each class.

### 3.1 | AndroR transcriptional activation assay

This assay allows for identification of chemical substances that activate the AR. Results are shown in Figure 2. The vehicle control containing only DMSO defines baseline activity set at 100% activity. Relative activity up to 130% represents inactive compounds, up to 160% relative activity classifies substances as partial agonists of the AR, and up to 200% as full agonists. Results are reported as % relative activity on a scale of 0% to 200% with DMSO defining 100% relative activity (baseline) and dihydrotestosterone defining 200% (full agonist). As expected, neither the GHS (which was added as a negative control) nor both SERMs showed any significant AR activation. In contrast, all of the AASs were full agonists with DHCMT being less active than STAN or MET, two of the most frequently detected steroids in doping control analysis.[26] The included SARMs were found to be at least partial agonists or fulfilled the criteria for full agonists. An interesting exception was LGD4, which demonstrated an AR activity substantially beyond all classical AASs. A relative activity >200% is rarely observed using the herein employed test, and triplicate repetition of the experiment corroborated the observation with relative activities determined between 240% and 280%. Bone and muscle anabolic properties of LGD4 were described in a randomized clinical trial in 2013, where increased lean body mass and decreased testosterone levels have been reported.[27] Since 2015, LGD4 has been among those SARMs being frequently detected in sports drug testing samples. Whether or not this indicates superior anabolic properties of LGD4 compared with other compounds remains unclear, and further information provided by complementary assays is required. Regarding its potential as CF, TB was found to fall between DHCMT and STAN and therefore should exhibit at least medium anabolic properties in humans.

### 3.2 | AndroR binding assay

The binding assay was used to complement the AndroR assay as it enables the determination of the binding capacity of a compound to the AR regardless of its agonistic or antagonistic properties. Figure 3 demonstrates the binding results correlated with the AR activation results, with most CFs showing good correlation. Both SERMs and the GHS proved to be inactive. PF represents an outlier that may be attributed to a different binding mode, but further investigations would be necessary to corroborate this finding. Interestingly, all AASs fall above the bisector line; that is, they are potentially stronger in activation than in binding to the AR, whereas TB falls below that line, demonstrating a reduced activation capacity. A similar behavior was noted for some of the SARMs. For example, ACP and AND show

**TABLE 3** Summary of analytical parameters for target analytes measured by GC/MS

| Analyte | RT | Precursor ion | Product ion |
|---|---|---|---|
| DHEA-D6 | 7.6 | 438 | 246 |
| | | 438 | 169 |
| DHEA | 7.6 | 432 | 237 |
| | | 432 | 169 |
| ADION | 8.1 | 430 | 181 |
| | | 430 | 169 |
| T-D3 | 8.2 | 435 | 330 |
| | | 435 | 209 |
| T | 8.2 | 432 | 209 |
| | | 432 | 181 |
| MeT | 9.0 | 301 | 169 |
| | | 301 | 143 |
| PREG | 9.6 | 445 | 157 |
| | | 157 | 141 |
| 17aPREG | 10.5 | 244 | 147 |
| | | 230 | 133 |
| 17aPROG | 11.0 | 316 | 193 |
| | | 301 | 169 |
| 11DEOXY | 12.2 | 301 | 169 |
| | | 230 | 147 |
| CORT-D4 | 13.3 | 636 | 235 |
| | | 636 | 147 |
| CORT | 13.3 | 632 | 234 |
| | | 632 | 191 |

*Note*: Listed are the retention times (RTs) and the applied precursor–product ion pairs (all measured at unit resolution). The dwell time was at 50 ms, and the collision energy was uniform at 30 eV.

exactly the same relative activity (Figure 2) but clearly separate in this comparison.

### 3.3 | Aromatase assay

A number of potential CFs under investigation did not alter aromatase activity (Figure 4). This included both SERMs (CLOM and TA), GHS, and several SARMs (MK0, MK6, TFM, AND, and LY), which might be interpreted as tissue selectivity. In contrast, most AASs strongly or moderately inhibit aromatase activity as observed with MSTEN, DHCMT, MET, and STAN, respectively. TB did not follow that pattern, and no aromatase inhibitory potential was recorded. Four SARMs (ACP, RAD, LGD4, and GSK) exhibited aromatase inhibitory effects, suggesting that some SARMs are not exclusively selective for the AR.

### 3.4 | Steroidogenesis assay

The human H295R adreno-carcinoma cell line represents an independently validated assay method to analyze effects of compounds on steroidogenesis in vitro.[13] It expresses the enzymes capable of producing the glucocorticoids and mineralocorticoids as well as the sex hormones. Each potential CF under investigation was subjected twice to the steroidogenesis assay, allowing analyses first with the LC/MS approach and then with GC/MS. The GC/MS results are considered as complementary information to the LC/MS-derived in vitro assay results. As the potential CFs under investigation differ significantly regarding their expected action on steroidogenesis, the results will be presented by each class of compounds to enhance readability and comparability. In order to improve traceability of the discussed results, a schematic of the steroidogenesis pathways is shown in Figure 5.

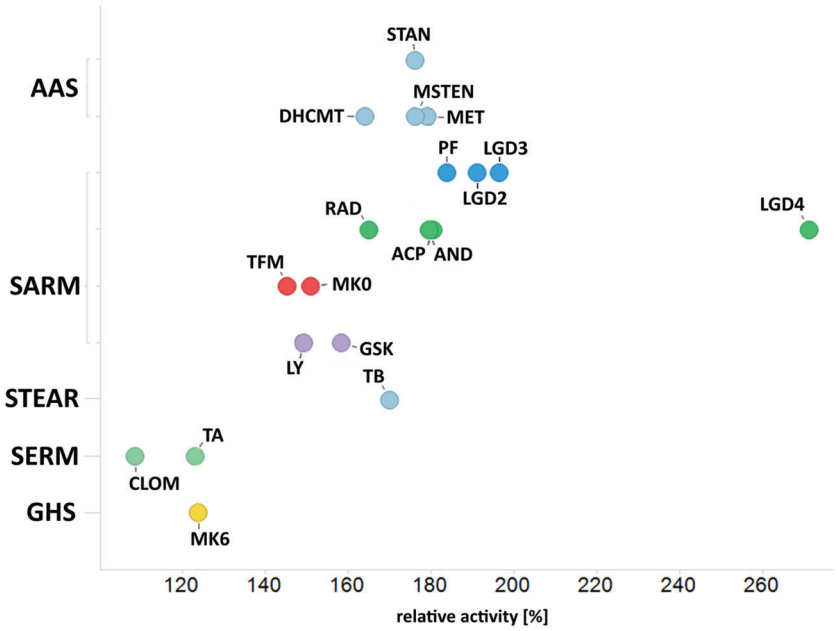


**FIGURE 2** Results obtained for the AR activation for each compound under investigation. Further information in the text

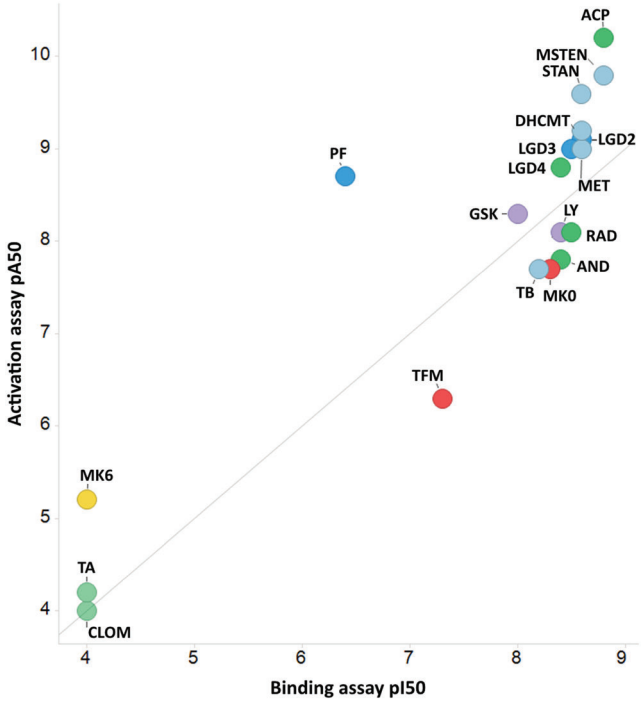


**FIGURE 3** Comparison of the activation and binding capacity of the androgen receptor for all investigated compounds. The line represents $x = y$. Results are reported as pI50 (negative base 10 logarithm of the IC50) for the binding assay and pA50 (negative base 10 logarithm of the concentration at 150% activity) for the activation assay. The value 4 is used to graphically represent compounds for which a pI50 or pA50 was not observed in the measured concentration range

### 3.4.1 | Growth hormone secretagogue

The first compound under consideration was added as a negative control—MK6. Occasionally referred to as ibutamoren mesylate, the drug candidate is still considered an investigational new drug without permission for human use. Effects and side effects described to date do not encompass any action on steroid metabolism.[28]

The assay did not respond in any significant way to MK6, as evidenced by both the LC/MS (Figure 6) and GC/MS (data not shown) results.

### 3.4.2 | Anabolic–androgenic steroids

Each steroid under investigation showed a strong impact on the steroidogenesis assay (Figure 7), and this was expected for the chosen subset of steroids as all of them belong to the class of AASs known to be misused in sports and are frequently detected.[23,24] Somehow surprising was the significantly diverse impact the different steroids had on the H295R cell line despite showing at least comparable results using the AndroR and aromatase activity assays. While STAN and MSTEN both strongly increased the production of PROG, it was decreased by DHCMT and, at high concentrations, by MET. T and CORT were generally decreased at higher concentrations with the exception of MET showing an intermediate increase in T. E2 and ES were decreased by the AASs except for STAN. This is in line with the results obtained for aromatase inhibition where STAN was less potent than the other steroids.

Literature on the impact of exogenous steroids on the excretion of urinary steroids is scarce, and owing to ethical considerations, no controlled administration trials have been conducted. Most of the relevant information has been derived from self-reported administrations or from population-based observations in bodybuilders or powerlifters.[2] Regarding STAN, one investigation reports the peculiar impact on the steroid profile.[29] Comparing two groups of bodybuilders, one administering only metenolone, the other metenolone and STAN, both could clearly be separated based on absolute urinary steroid concentrations and concentrations ratios. The combined administration resulted in a significantly larger suppression of endogenous steroid production and in increased concentration ratios for T/E

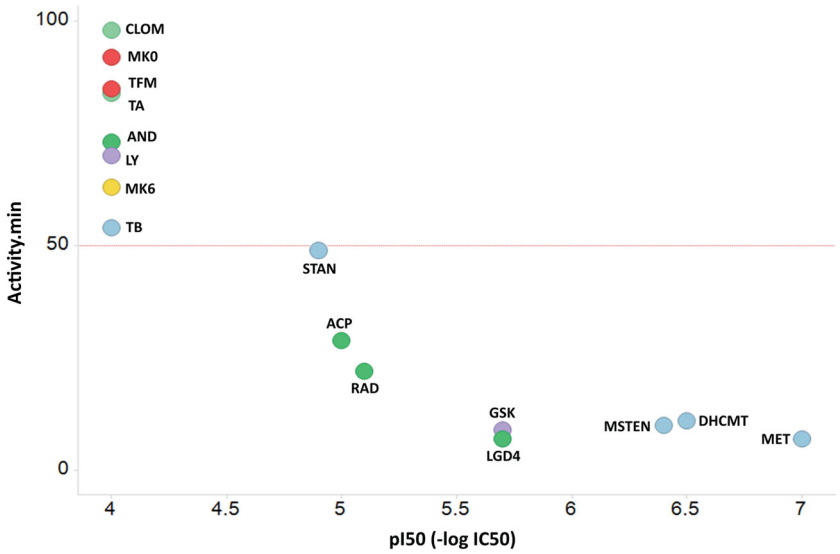


**FIGURE 4** Results obtained for the aromatase assay. Results are reported as pI50 (negative base 10 logarithm of the IC50). The value 4 is used to graphically represent compounds for which a pI50 was not observed in the measured concentration range. Activity.min is the lowest observed enzyme activity in the dose–response curve, where 100% activity corresponds to fully functional enzyme and 0% corresponds to fully inhibited enzyme. LGD2, LGD3, and PF could not be measured owing to the extensive autofluorescence of these compounds

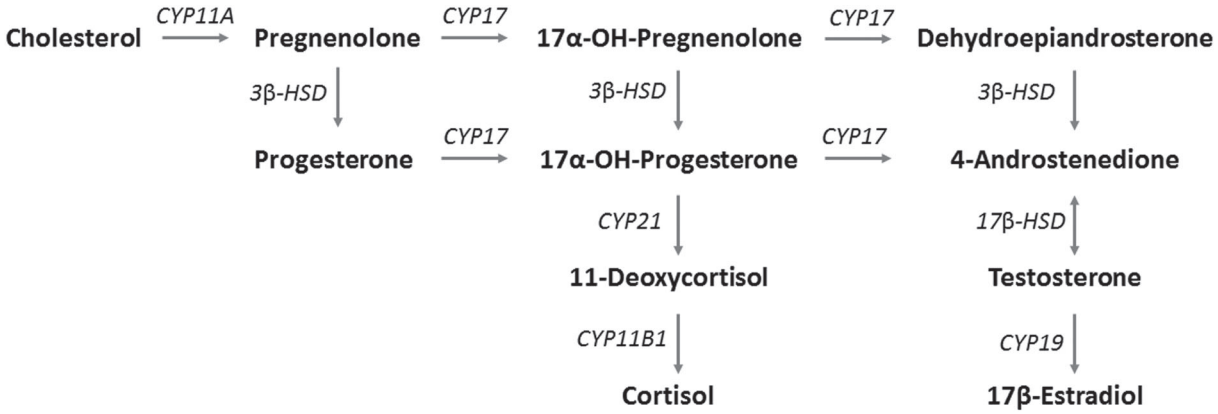


**FIGURE 5** Steroidogenic pathway in H295R cells (adopted from OECD[13]). CYP stands for cytochrome P450, and HSD for hydroxysteroid dehydrogenase

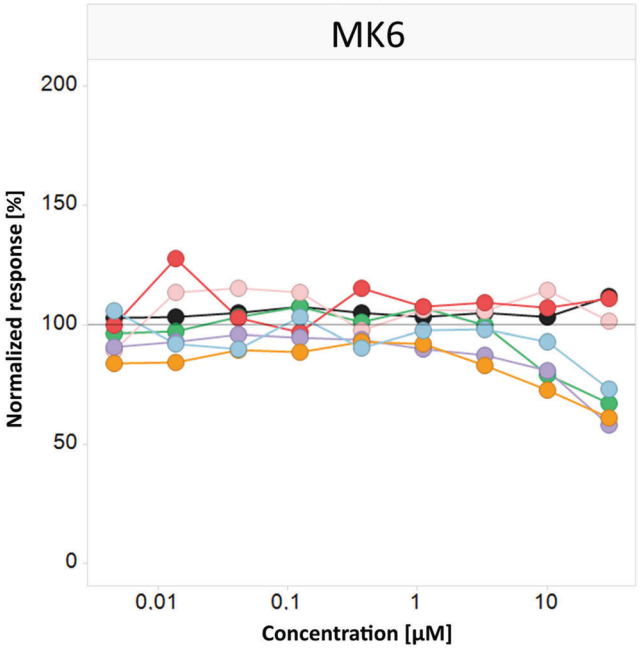


**FIGURE 6** Concentration-dependent response of the steroidogenesis assay on MK6. Color codes: light blue, testosterone; purple, 17α-OH-progesterone; orange, progesterone; red, estrone; pink, estradiol; green, cortisol; and black, the cell viability. Results were normalized to the dimethyl sulfoxide (DMSO) vehicle control

and 11-OH-androsterone (11OHA) divided by 11-OH-etiocholanolone (11OHE). A different mode of action for both steroids on the human steroidogenesis was assumed but not further investigated. The assumption raised by Saartok et al. that STAN has only a weak interaction with the AR was not corroborated by the herein obtained results (Figures 2 and 3).[30]

For MET in particular and other AASs in general, a strong decrease in endogenous steroid production has been reported.[31–33] Steroids mainly derived from testicular production like T and E, but also steroids produced in the adrenal glands (corticosteroids and their main metabolites 11OHA and 11OHE) were affected, pointing towards a general mode of action of AASs as CFs. Major enzymatic steps in adrenal (and H295R) steroid production include 3β-hydroxysteroid-dehydrogenase/$\Delta^{4,5}$-isomerase, 17,20-lyase and 5β-reductase.[34,35] The latter could not be further investigated with the steroidogenesis assay as the counterpart of 5β-reductase, the 5α-reductase, is mainly found in peripheral tissue, and differences in the steroid profile for 5α- and 5β-steroids (e.g., the influence on 11OHA/11OHE) will not be solely due to an impact on steroidogenesis even if the 11OHA/11OHE ratio is mainly affected by cortisol production and metabolism. Other enzymatic reactions however can be monitored by measuring different target analytes with GC/MS. The combination of ADION, DHEA, 17OH-PROG, and 17OH-PREG in different combinations allowed for the investigation of both the isomerase and lyase activities. In accordance to conventional steroid profile analyses, steroid abundance ratios were applied. The ratios of ADION/DHEA and 17OH-PROG/17OH-PREG show the influence on the $\Delta^{4,5}$-isomerase activity, and alterations of the ratios of DHEA/17OH-PREG and ADION/17OH-PROG indicate a compound's impact on the 17,20-lyase activity.

For DHCMT (Figure 8), a rather clear picture is obtained: both ratios illustrating the activity of $\Delta^{4,5}$-isomerase (ADION/DHEA and 17OH-PROG/17OH-PREG) are strongly influenced by increasing concentrations; that is, the conversion of 5-ene-steroids to 4-ene-steroids is significantly downregulated. In parallel, the 17,20-lyase activity is upregulated as demonstrated by the increasing ratios of DHEA/17 OH-PREG and ADION/17OH-PROG. The counterexample is STAN (Figure 8) with contradictory results found for the $\Delta^{4,5}$-isomerase with one ratio increasing and one decreasing, and incongruent results for the 17,20-lyase with one ratio being constant whereas the other decreases. Taking a look at the absolute concentrations showed that mainly ADION was decreased. A similar result was obtained for MSTEN, whereas for MET, the concentration of DHEA was increased (data not shown). Unfortunately, a direct relationship with suppressions in the steroid profile cannot be established from these results, even within the same class of closely related compounds.

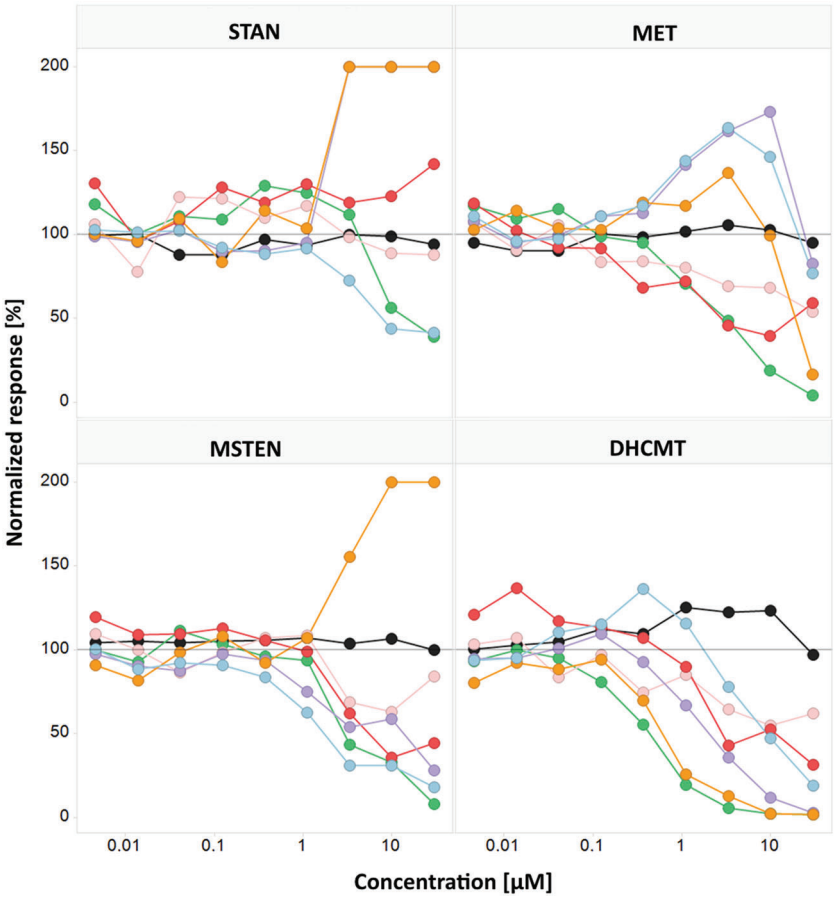


**FIGURE 7** Concentration-dependent response of the steroidogenesis assay on anabolic–androgenic steroids (AASs). Color codes: light blue, testosterone; purple, 17α-OH-progesterone; orange, progesterone; red, estrone; pink, estradiol; green, cortisol; and black, the cell viability. Results were normalized to the dimethyl sulfoxide (DMSO) vehicle control

A larger subset of AASs has recently been investigated using the steroidogenesis assay by another group corroborating the diverse findings within the same class of substances.[36] For two compounds, a direct comparison of the results was possible, albeit that this study only tested on one concentration level (1 µM) against a negative control. Nevertheless, STAN also showed a significant upregulation of PROG and 17OH-PROG together with a downregulation of ADION. Additionally, an upregulation of 11CORT was reported, which was also measurable in our study (130% at 1 µM and 180% at 4 µM) but which was not considered significant, as at higher concentrations of STAN the concentration-dependent increase was not inverted and the concentration returned to starting values. For the other AASs under investigation, MET, a similar slight increase in PROG and a stronger increase in 17OH-PROG were determined.

### 3.4.3 | Selective estrogen receptor modulators

The results obtained for both SERMs (CLOM and TA) were highly comparable with both showing a concentration-dependent decrease of PROG, 17OH-PROG, CORT, and T, whereas E2 and ES were not affected. The steroidogenesis assay showed a strong decrease in cell viability for both SERMs at 30 µM. The complementary GC/MS analysis corroborated the finding for CORT and showed additionally a decrease in ADION and 11CORT.

Despite these clear results, the comparison with the scarce data of human excretion studies is not straightforward. For both CLOM and TA, a slight increase in T and E concentrations was observed in male but not in female volunteers after multiple administrations, whereas other markers of the steroid profile were not affected.[8] These results were corroborated by another study on CLOM.[37] Here also, the concentrations of T and E were found to increase slightly over time, whereas E2 and ES showed a strong increase. These first results suggest that the impact on the adrenal steroid production does not prevail after oral administrations of TA or CLOM in humans. Further investigations encompassing relevant urinary steroids like 11OHA and 11OHE would be beneficial for a final evaluation.

### 3.4.4 | Selective AR modulators

The results for the steroidogenesis assay were as diverse as the chemical structures of the investigated subgroup of SARMs. No significant impact was noted for LGD2, LGD3, PF, GSK, and LY (data not shown). The response of the assay towards MK0, TFM, and LGD4 was comparable as demonstrated in Figure 9. All induced a concentration-dependent decrease of PROG, 17OH-PROG, and CORT and some minor changes in T. ACP reduced CORT concentrations and increased 17OH-PROG, whereas AND and RAD exhibited weak effects on T and PROG. The GC/MS-based determinations also did not show any

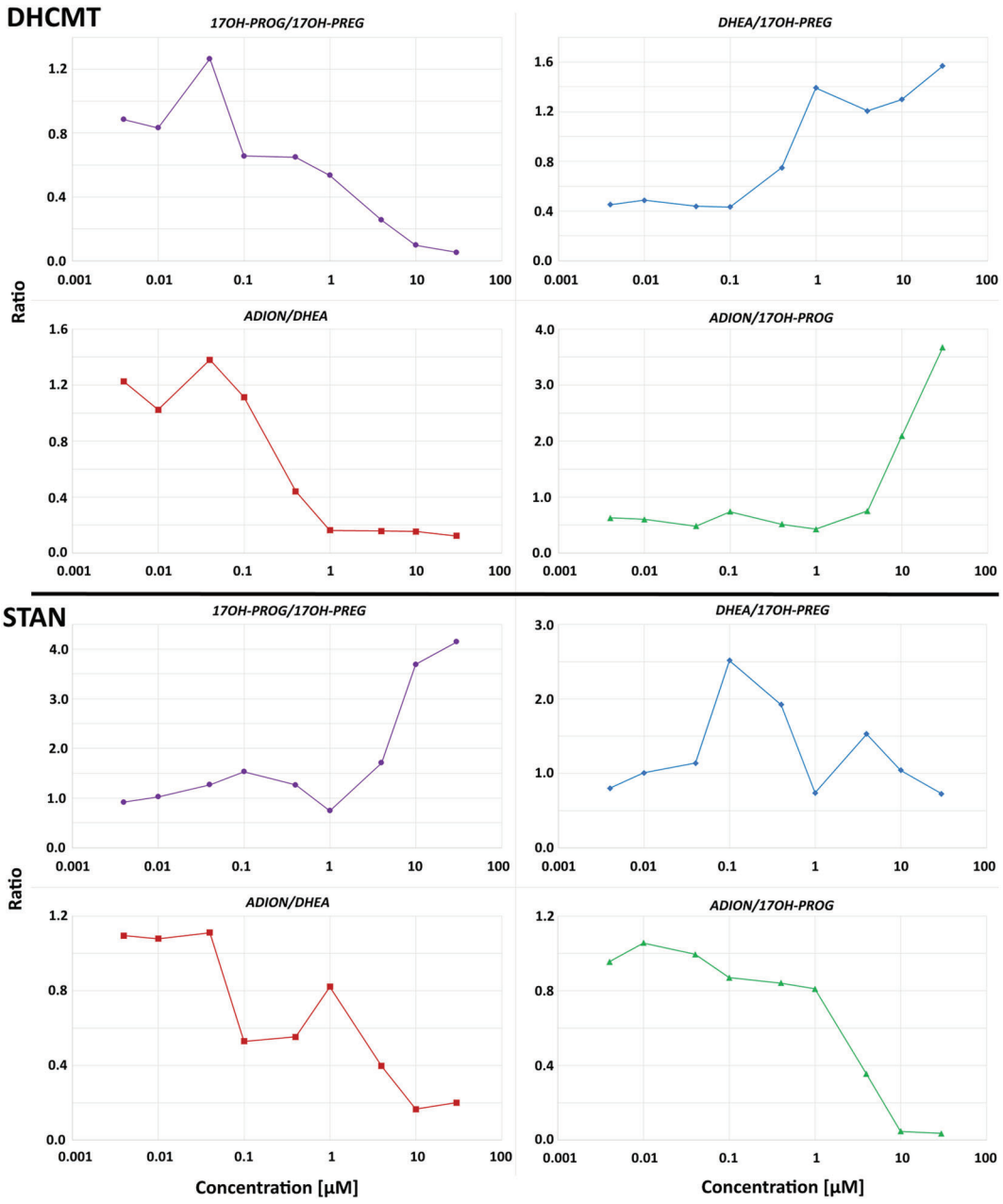


**FIGURE 8** Concentration ratios of GC/MS-measured steroidal analytes, determined at different drug concentrations using the steroidogenesis assay. Top part, oral turinabol; bottom part, stanozolol. Further information in the text

significant changes except for TFM, which showed an impact on the $\Delta^{4,5}$-isomerase with increased concentration of DHEA and 17OH-PREG and decreased concentration of ADION and 17OH-PROG. It was expected that different SARMs would show comparable results because of their identical chemical core structure, but no correlations were found corroborating this hypothesis. Each SARM induced a highly individualized response, and some compounds did not show an exclusive action on the AR within this study.

The above-mentioned study on AASs also investigated LGD2 and LGD4.[36] For both SARMS, no significant effect on the concentrations of the steroidogenesis assay was found, which was in line with our findings taking into account that here only a concentration of 1 µM was investigated, whereas the effects noted for LGD4 were mainly found at higher concentration levels.

### 3.4.5 | Selective tissue estrogenic activity regulator

Most of the publications on TB deal with medical aspects rather than with an impact on steroidogenesis.[21,38,39] Only one publication considered the possible impact of TB on human steroid production and compared TB with other common hormone-replacement therapies.[40] The effects described for TB encompassed constant plasma levels for

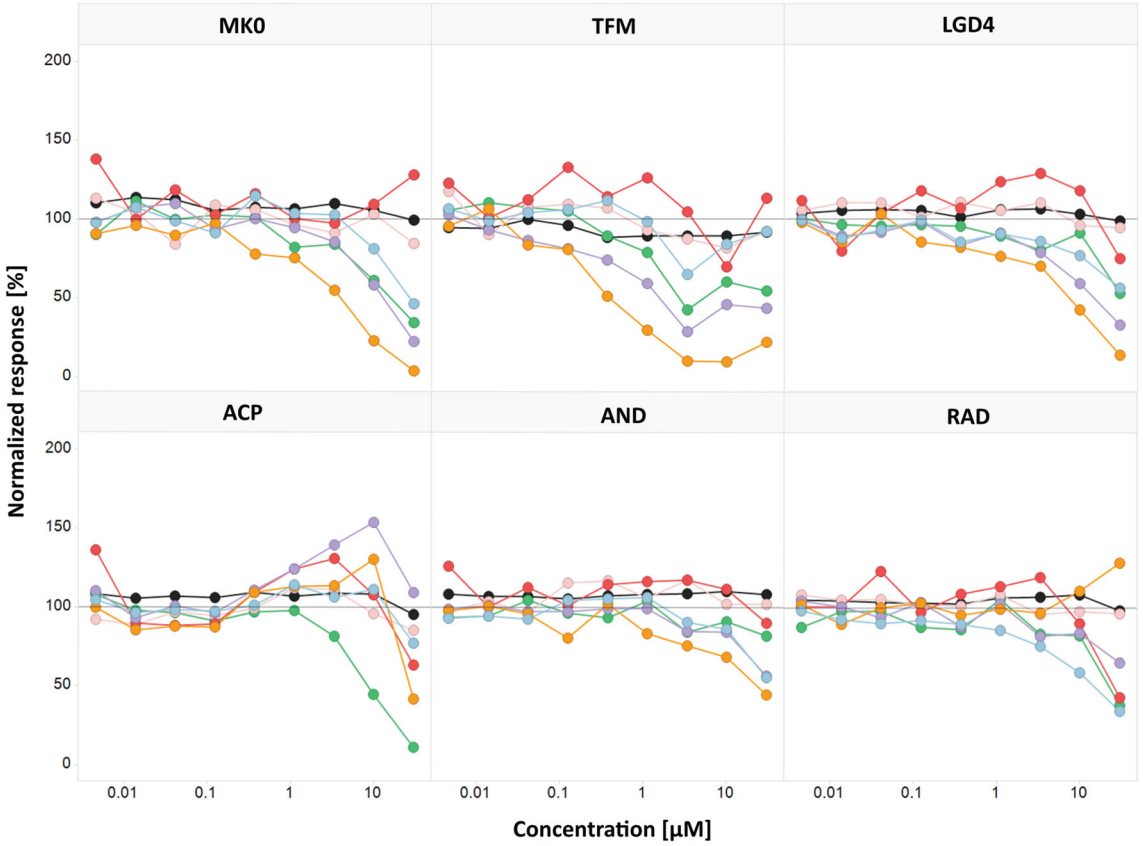


**FIGURE 9** Concentration-dependent response of the steroidogenesis assay on selected SARMs. Color codes: light blue, testosterone; purple, 17α-OH-progesterone; orange, progesterone; red, estrone; pink, estradiol; green, cortisol; and black, the cell viability. Results were normalized to the dimethyl sulfoxide (DMSO) vehicle control

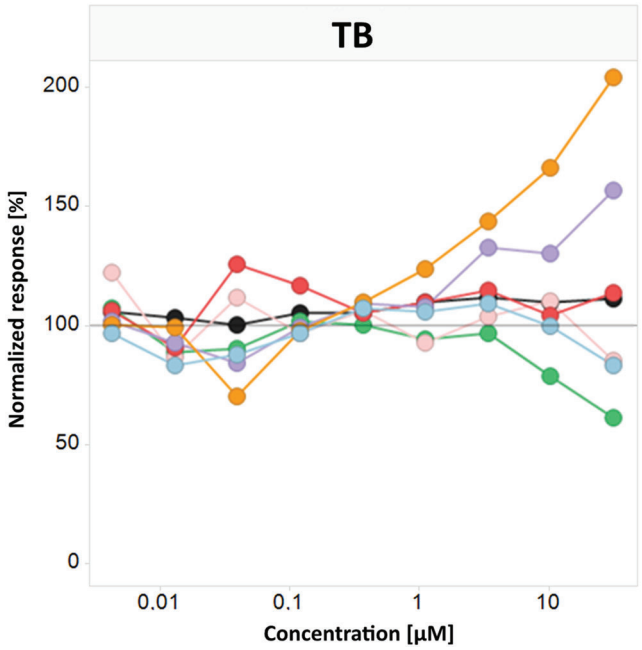


**FIGURE 10** Concentration-dependent response of the steroidogenesis assay on TB. Color codes: light blue, testosterone; purple, 17α-OH-progesterone; orange, progesterone; red, estrone; pink, estradiol; green, cortisol; and black, the cell viability. Results were normalized to the dimethyl sulfoxide (DMSO) vehicle control

E2 and DHEA, a significant increase in PROG, and a slight decrease in CORT studied over a period of 12 months. These results are well reflected by the steroidogenesis assay as shown in Figure 10. Besides an increase in PROG, 17OH-PROG was also found elevated, and for higher concentrations, a decrease in CORT is visible. The GC/MS results showed an additional slight increase in PREG and constant levels for DHEA. Regarding the plasma levels of different endogenous steroids, the assay was obviously able to emulate the human metabolism.

## 4 | CONCLUSION

The identification of CFs with an impact on the steroid profile is an issue for sports drug testing. Only a few compounds have been carefully investigated, whereas for the vast majority of substances, no reliable data exist. Additionally, for many of the compounds relevant for doping control analysis, a controlled administration trial encompassing human subjects is not feasible as they are classified as nonapproved substances. Therefore, the usefulness of in vitro assays was investigated with an emphasis on the steroidogenesis assay employing H295R cells.

The in vitro approach including tests of AR activation and binding capacity together with aromatase activity and steroidogenesis assays

enabled a comprehensive overview of the possible actions of a compound on the cellular level. The impact found at this level is unfortunately not necessarily directly transferable to influences found on the steroid profile. This has been demonstrated for the AASs, all showing a significant but diverse response in the steroidogenesis assay; however, the prolonged administration of these compounds always results in a strong, and to a large extent, comparable suppression of the steroid profile. Here, obviously other mechanisms like the negative feedback regulation for steroid production prevail over impact on cell function. These feedback regulations driven by the hypothalamic–pituitary–adrenal axis cannot be emulated by the combination of different assays employed in this study.

Regarding the SARMs under investigation, no direct comparison with administration trials was possible. Results obtained here suggest that five of the SARMs (LGD2, LGD3, PF, GSK, and LY) will most probably not have a strong impact on the steroid profile, whereas for the other six (MK0, TFM, LGD4, ACP, AND, and RAD), a confounding influence cannot be excluded.

The two investigated SERMs (CLOM and TA) showed a significant impact on steroid production in the adrenal cell line, which was not reflected by previously reported changes in the urinary steroid profile.[8,37] For a sound interpretation, it is necessary to consider that the focus of the excretion studies regarding the influence on the steroid profile was on T and E and that relevant steroids of adrenal production like 11OHA or 11OHE were not determined. Therefore, it is not possible to draw a final conclusion regarding the potential of the steroidogenesis assay for these compounds.

Of interest is the STEAR TB and the question of whether this compound comprising a steroidal backbone may be considered a CF. Besides androgenic properties, TB triggered a response of the steroidogenesis assay especially at elevated concentrations. As no data regarding the impact on the urinary steroid profile were available, the results were compared with changes found in several plasma steroids, and the identified congruency was surprisingly high.[40]

The compound added as a negative control (MK6) did not show any significant impact on any of the in vitro assays.

Summarizing these finding suggest that our in vitro assay approach does enable us to predict if a compound may have an impact on the steroid profile used in sports drug testing. However, it will be hard to derive which subset of profiled steroids could be impacted as insight derived from the in vitro assays will be limited because neither the physiological regulatory mechanisms of the hypothalamic–pituitary–gonadal axis, nor absorption, distribution, metabolism, and excretion of the compounds can be successfully mimicked in the in vitro assay. Expanding the analytical profile of the steroidogenesis assay including monitoring more steroids should further improve the capability of this assay to predict possible impact on human steroid metabolism.

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